Large Granular Lymphocytes Have a Promoting Activity on Human Peripheral Blood Erythroid Burst-Forming Units

By V. Pistoia, R. Ghio, A. Nocera, A. Leprini, Angela Perata, and M. Ferrarini

Peripheral blood mononuclear cells were fractionated according to the expression of a variety of surface markers, and the fractions obtained were tested for erythroid burst-forming unit (BFU-E) colony formation. BFU-Es were detected in the HLA-DR<sup>+</sup> non-T cell fraction, but gave rise to optimum colony numbers only in the presence of a nonadherent, relatively radioresistant cell. This accessory cell was found among the HLA-DR<sup>+</sup> non-T, non-B cells, a fraction that was particularly enriched in large granular lymphocytes (LGLs). Experiments carried out to assess directly the surface markers of the accessory cell revealed that BFU-Es represent more immature progenitors than CFU-Es.<sup>5</sup> Presently there is consensus that BFU-Es represent more immature progenitors than CFU-Es.<sup>5</sup>9

In vitro studies have demonstrated that T cells<sup>10</sup>-<sup>15</sup> monocytes,<sup>11,16-19</sup> and some as yet unidentified cells within the null cell subset<sup>20</sup> exert regulatory control on peripheral blood BFU-Es. Bone marrow BFU-E colony formation is also regulated in vitro by a number of factors. Furthermore, they give rise to colonies of different size and type.<sup>3,7</sup> Presently there is consensus that BFU-Es represent more immature progenitors than CFU-Es.<sup>5</sup>9

Since previous investigations had demonstrated that BFU-Es have HLA-DR surface antigens,<sup>23</sup> we fractionated peripheral blood MNCs according to the expression of these markers in an attempt to obtain suspensions highly enriched for BFU-Es. These experiments led to the observation that in order to develop into colonies, BFU-Es require the help of a non-T, non-B HLA-DR<sup>+</sup> accessory cell capable of releasing factor(s) with a burst-promoting activity. This accessory cell has been identified as belonging to a subset of granular cells, generally called large granular lymphocytes (LGLs).<sup>26</sup>28

MATERIALS AND METHODS

Cell Fractionation Procedures

Leukocyte-rich buffy coats were obtained from the plateletpheresis of 300 to 500 mL of blood, and MNCs were separated on Ficoll-Hypaque density gradients. Monocytes were partially removed by adherence on plastic Petri dishes. T cells were separated from adherent cell-depleted MNCs by rosetting with neuraminidase-treated sheep erythrocytes (E) followed by fractionation on two subsequent Ficoll-Hypaque density gradients.<sup>23</sup> Indicator cells were removed from E-rosetting cells (T cells) by hypotonic lysis. Nonrosetting cells (non-T cells) were re-rosetting and depleted of the remaining T cells through another Ficoll-Hypaque gradient.<sup>24</sup> Cells with receptors for the Fe portion of IgG (FcR) were detected by rosetting with ox red blood cells coated with rabbit IgG antibody (EA).<sup>25</sup> The rosetting cells (FcR<sup>+</sup> cells) were separated from nonrosetting cells (FcR<sup>-</sup> cells) on two Ficoll-Hypaque gradients.<sup>30</sup> Indicator cells were removed from rosetting cells by hypotonic lysis.

Cells that expressed surface HLA-DR antigens were detected by rosetting with ox red blood cells coated with the PTF 29/12 anti-HLA-DR monoclonal antibody.<sup>26</sup> This murine monoclonal antibody was purified from ascitic fluids on a Sephadex G 200 column, dialyzed against saline, and coupled to ox red blood cells according to the technique of Ling et al.<sup>27</sup> Cells were rosetted at 4 °C.<sup>32</sup> Rosettes (HLA-DR<sup>+</sup> cells) were separated from nonrosetting cells (HLA-DR<sup>-</sup> cells) on Ficoll-Hypaque density gradients.

In all of the above experiments, the rosette-enriched fractions contained a minimum of 94% rosetting cells and the rosette-depleted fractions never had more than 4% rosettes.

T cells never contained more than 2% cells with surface immunoglobulin (sIg) or HLA-DR antigens. Additional details on the above cell fractions and others described in this article are given in Table I.
and from Dr G. Damiani, respectively. An anti-monocyte-Calif) different sources. subpopulations were obtained from globulin (polyvalent) serum conjugated with fluorescein isothiocyanate! Mouse monoclonal antibodies to human mononuclear cell reagents (OKT3, OKT4, and OKT8) were from Ortho Pharmaceutical Corp. (HNK1) which was a generous gift of Drs M. Cooper and T. Abo, and Leu 11b, which was purchased from Beckon Dickinson. All of the above reagents were used in indirect immunofluorescence with a FITC-conjugated rabbit F(ab')2 anti-mouse immunoglobulin (polyvalent). Preparations were observed under a Leitz (Wetzlar, FRG) Orthoplan fluorescence microscope. The percent of positive cells was calculated on counts of a minimum of 200 cells per preparation. Cell surface marker analysis of cells that had been fractionated as described previously was carried out immediately after the separation procedures or following an 18-hour incubation in RPMI containing 10% FCS at 37 °C. Preliminary comparative studies did not disclose any difference between the two procedures.

Cytotoxic activity of LGLs was measured by the 51Cr release assay, according to a modification of the technique of Timonen et al.23 Briefly, seven different Percoll (Pharmacia, Uppsala, Sweden) concentrations were prepared by diluting the 100% stock solution with RPMI 1640 supplemented with 10% fetal calf serum (FCS, Gibco-Biocult, Glasgow, Scotland). The concentrations ranged from 40% to 55%, and each of them differed from the next by 2.5%. For each concentration, osmolarity and density were both checked and adjusted when necessary.27 The gradient was prepared by layering 2-mL aliquots of the different Percoll solutions in 15-mL conical test tubes, starting with the solution with the highest density (ie, 55%). Then 5 x 10³ lymphocytes were resuspended in 1 mL of RPMI FCS and placed on the top of the gradient. The tubes were centrifuged at 550 g for 30 minutes at room temperature. Seven cell fractions were recovered and pooled and depleted of cells forming E rosettes. Fractions 2 and 3 from Percoll gradients were pooled and depleted of cells forming E rosettes.29 Cells with surface Ig receptors for sheep red blood cells or FcR were detected by staining with a rabbit F(ab')2 anti-human immunoglobulin (polyvalent). Preparations were observed under a Leitz (Wetzlar, FRG) Orthoplan fluorescence microscope. The percent of positive cells was calculated on counts of a minimum of 200 cells per preparation. Cell surface marker analysis of cells that had been fractionated as described previously was carried out immediately after the separation procedures or following an 18-hour incubation in RPMI containing 10% FCS at 37 °C. Preliminary comparative studies did not disclose any difference between the two procedures.

Cytotoxicity and Criteria for LGL Identification

LGLs present in the different cell fractions were identified by staining the cells with Giemsa or by the cytochemical localization of α-naphthyl acid esterase (ANAЕ) or acid phosphatase (AP) as

**Table 1. Characterization of Fractionated Non-T Cells and of Percoll-Purified LGLs**

<table>
<thead>
<tr>
<th>Surface marker</th>
<th>HLA-DR non-T Cells*</th>
<th>HLA-DR* non-T Cells*</th>
<th>Percoll-Purified LGLs*†</th>
</tr>
</thead>
<tbody>
<tr>
<td>FcR</td>
<td>82 ± 6</td>
<td>35 ± 6</td>
<td>69 ± 5</td>
</tr>
<tr>
<td>E</td>
<td>&lt;2</td>
<td>&lt;1</td>
<td>&lt;3</td>
</tr>
<tr>
<td>slg</td>
<td>&lt;3</td>
<td>60 ± 7</td>
<td>&lt;5</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>&lt;3</td>
<td>90 ± 7</td>
<td>&lt;5</td>
</tr>
<tr>
<td>OKT3</td>
<td>&lt;3</td>
<td>&lt;1</td>
<td>&lt;3</td>
</tr>
<tr>
<td>OKT4</td>
<td>&lt;3</td>
<td>&lt;1</td>
<td>&lt;3</td>
</tr>
<tr>
<td>OKT8</td>
<td>13 ± 2</td>
<td>&lt;3</td>
<td>11 ± 1</td>
</tr>
<tr>
<td>OKM1</td>
<td>68 ± 5</td>
<td>30 ± 5</td>
<td>56 ± 5</td>
</tr>
<tr>
<td>Leu 7</td>
<td>74 ± 6</td>
<td>&lt;3</td>
<td>60 ± 5</td>
</tr>
<tr>
<td>Leu 11</td>
<td>78 ± 7</td>
<td>&lt;3</td>
<td>ND</td>
</tr>
</tbody>
</table>

Cytochemical marker‡↓

<table>
<thead>
<tr>
<th>Acid hydrolases</th>
<th>HLA-DR non-T Cells*</th>
<th>HLA-DR* non-T Cells*</th>
<th>Percoll-Purified LGLs*†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Paranuclear</td>
<td>81 ± 8</td>
<td>70 ± 6</td>
<td></td>
</tr>
<tr>
<td>2. Dot-like</td>
<td>&lt;3</td>
<td>&lt;3</td>
<td>&lt;3</td>
</tr>
<tr>
<td>3. Diffuse</td>
<td>&lt;3</td>
<td>29 ± 3</td>
<td>&lt;3</td>
</tr>
<tr>
<td>NK activity§</td>
<td>1,058 ± 304</td>
<td>45 ± 15</td>
<td>1,300 ± 330</td>
</tr>
</tbody>
</table>

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LGLs were also isolated from adherent cell-depleted MNCs on a discontinuous Percoll density gradient, according to a modification of the technique of Timonen et al.23 Briefly, seven different Percoll (Pharmacia, Uppsala, Sweden) concentrations were prepared by diluting the 100% stock solution with RPMI 1640 supplemented with 10% fetal calf serum (FCS, Gibco-Biocult, Glasgow, Scotland). The concentrations ranged from 40% to 55%, and each of them differed from the next by 2.5%. For each concentration, osmolarity and density were both checked and adjusted when necessary.27 The gradient was prepared by layering 2-mL aliquots of the different Percoll solutions in 15-mL conical test tubes, starting with the solution with the highest density (ie, 55%). Then 5 x 10³ lymphocytes were resuspended in 1 mL of RPMI FCS and placed on the top of the gradient. The tubes were centrifuged at 550 g for 30 minutes at room temperature. Seven cell fractions were recovered and washed extensively in phosphate-buffered saline (PBS). In each fraction, the cell viability was 90% or more, as determined by trypan blue exclusion. Fractions 2 and 3, which contained the highest proportion of LGLs, were pooled and depleted of cells forming E rosettes. Fraction 1 contained 90% or more monocytes, whereas the remaining fractions contained almost exclusively small lymphocytes as assessed by morphology or cytochemistry (see Cytotoxicity and Criteria for LGL Identification). Figure 1 summarizes the cell fractionation procedures that were employed.

**Cell Surface Marker Analysis**

Cells with receptors for sheep red blood cells or FcR were detected by rosette techniques as described previously.29 Cells with surface Ig were detected by staining with a rabbit F(ab')2, anti-human immunoglobulin (polyvalent) serum conjugated with fluorescein isothiocyanate.30 Mouse monoclonal antibodies to human mononuclear cell subpopulations were obtained from different sources. Anti-T cell reagents (OKT3, OKT4, and OKT8) were from Ortho Pharmaceutical Corp. (Raritan, NJ). Anti-HLA-DR antibodies (HLA-DR or PTF 29/12) were obtained from Becton Dickinson (Sunnyvale Calif) and from Dr G. Damiani, respectively. An anti-monocyte-macrophage monoclonal reagent that also stains natural killer (NK) cells (OKM1) was purchased from Ortho Pharmaceutical Corp.31 Two LGL-specific monoclonal antibodies were used, Leu 7, (HNK1) which was a generous gift of Drs M. Cooper and T. Abo, and Leu 11b, which was purchased from Beckon Dickinson. All of the above reagents were used in indirect immunofluorescence with a FITC-conjugated rabbit F(ab')2 anti-mouse immunoglobulin (polyvalent). Preparations were observed under a Leitz (Wetzlar, FRG) Orthoplan fluorescence microscope. The percent of positive cells was calculated on counts of a minimum of 200 cells per preparation. Cell surface marker analysis of cells that had been fractionated as described previously was carried out immediately after the separation procedures or following an 18-hour incubation in RPMI containing 10% FCS at 37 °C. Preliminary comparative studies did not disclose any difference between the two procedures.

**Cytotoxicity and Criteria for LGL Identification**

LGLs present in the different cell fractions were identified by staining the cells with Giemsa or by the cytochemical localization of α-naphthyl acid esterase (ANAЕ) or acid phosphatase (AP) as

![Fig 1. Schematic representation of the methods used for cell fractionation.](image-url)
previously reported. In Giemsa-stained preparations, LGLs appear like medium-sized lymphocytes with abundant cytoplasm and typical azurophilic granules. In the preparations stained for ANAE, LGLs display a granular pattern of the enzyme activity. In contrast, T cells show a dot-like staining and B cells are mostly negative with the exception of a few, heavily stained plasma cells. Monocytes are heavily stained with the reaction product localized in large and confluent granules. A staining pattern similar to that of ANAE is observed with AP, except that LGLs are more strongly stained and T cells do not always show the dot-like pattern. The two cytochemical techniques can, therefore, be considered as complementary. The various proportions of cells in the different preparations were determined by counting a minimum of 300 cells and classifying them according to their cytochemical properties. In some experiments, in order to have a further check on the proportion of monocytes contaminating the various fractions, peroxidase (PO) staining was performed.

Cytotoxicity Assays

Cell cytotoxicity with monoclonal antibodies and complement was carried out according to the method of Zarling et al. Briefly, cells were suspended at a concentration of $1 \times 10^7$/mL in RPMI 1640 (Gibco-Biocult) medium, which contained OKT4 or OKT8 (at a final dilution of 1:100), OKM1 (at a final dilution of 1:20), Leu 7 (at a final dilution of 1:10), or Leu 11 (at a final dilution of 1:50) monoclonal antibodies or no antibody (control). After one-hour incubation on ice, nontoxic rabbit complement (Pel-Freeze, Rogers, Ark) was added at a final dilution of 1:2 in RPMI 1640. The cells were incubated at 37°C for one hour, washed twice, resuspended in fresh complement, and incubated again at room temperature for 30 minutes. Dead cells were removed by centrifugation of the cell suspension on Ficol-Hypaque gradients. When this procedure was not feasible, owing to the small cell numbers obtained, dead cells were detected by trypan blue staining and excluded from the counts carried out for the determination of cell concentration. In order to assess the efficiency of complement-mediated cytolysis, the proportion of dead cells in the suspensions treated with a given monoclonal antibody was determined by staining with trypan blue or ethidium bromide and compared to that of the cells stained by the same antibody in immunofluorescence. In the preparations used, complement-mediated lysis killed at least 80% of the cells that stained in immunofluorescence.

NK activity was measured in short-term assays using $^{51}$Cr-labeled K562 target cells as described previously.

Cell Irradiation

Cell suspensions at a concentration of $1 \times 10^6$ cells/mL were irradiated with gamma radiation (Co$^{60}$) at incremental doses (up to 5,000 rads) and washed twice in RPMI 1640.

Cell Cultures

Different peripheral blood cell fractions were tested for erythroid burst colony formation according to the method of Iscove. Briefly, 2 x $10^7$ cells were resuspended in a final volume of 1 mL of a mixture containing α-medium (Gibco, Biocult), 0.8% methylcellulose (Dow Chemical Co, Midland, Mich), 30% FCS (Gibco, Biocult), 10 $^{-4}$ mol/L α-thyoglicerol, 1% penicillin-streptomycin solution, and 2 IU erythropoietin (Connaught Labs, Toronto, Ontario). The plates were incubated at 37°C in 95% air and 5% CO2, and scored for colonies after 12 days. The erythroid nature of the colonies was ascertained by staining with benzidine in situ. Only those benzidine-positive colonies that contained a minimum of 50 cells were counted.

Culture Supernatants

Culture supernatants were prepared as follows. LGLs were purified by Percoll density gradients as above, resuspended at a concentration of 1 to 2 x $10^6$ cells/mL in RPMI 1640 containing 10% FCS, and cultured in 24-well plates for 48 hours. T cells were purified by E rosetting and were cultured under the same conditions as LGLs in the presence or absence of phytohemagglutinin (PHA-P, 1:1,000 vol/vol). Adherent cells (monocytes) were cultured for 48 hours in plastic flasks as previously described. The supernatants from all of the cultures were subsequently collected and stored at –20°C.

RESULTS

Identification of a Non-T Cell Capable of Promoting BFU-E Colony Formation

When unfractionated MNCs were cultured under optimal conditions, BFU-E colonies appeared after nine to ten days, reached a peak after 12 to 14 days, and thereafter began to degenerate. Most colonies were composed of large groups of 400 to 500 well-hemoglobinized cells containing two or more subcolonies, whereas a minority of them were smaller and appeared as single clusters of 50 to 200 cells. When MNCs were separated into T and non-T cells, colonies appeared in the non-T cell fraction only and were more numerous than in unseparated cells. In agreement with previous data, these colonies developed with the same kinetics as described previously, but were smaller, with a predominance of clusters containing 50 to 200 cells (data not shown).

Non-T cells were separated into HLA-DR+ and HLA-DR- cells, and the two fractions were tested for BFU-E colony formation. Small numbers of colonies were found occasionally in the HLA-DR+ cell fraction. Readition of HLA-DR+ to HLA-DR- cells (at a 1:1 ratio) increased the colony numbers to values comparable to those of unfractionated non-T cells. These findings, summarized in Table 2, suggest that optimal colony formation required the cooperation of HLA-DR- and HLA-DR+ cells. In order to identify the fraction containing the erythroid precursors, the following experiments were carried out. Non-T cells were separated into HLA-DR+ and HLA-DR- cells, and portions of each cell fraction were irradiated (3,000 rads) before being admixed with nonirradiated cells of the other fraction. Irradiation of HLA-DR+ cells virtually abrogated colony formation, whereas irradiation of HLA-DR- cells caused an approximately 50% decrease in the colony numbers (Table 2). A complete inhibition of BFU-E colonies was observed following irradiation of HLA-DR- non-T cells with 5,000 rads (data not shown). These data indicate that BFU-E were in the HLA-DR+ non-T cells and that they could develop into colonies in the presence of a relatively radioresistant HLA-DR- cell. Figure 2 shows a titration of the capacity of HLA-DR+ non-T cells of
supporting optimal BFU-E colony formation. BFU-E colonies increased progressively with the concentration of HLA-DR cells to reach a plateau at a ratio of HLA-DR/EILA-DR cells of approximately 1:1. The increase of HLA-DR cell concentration caused a concomitant change in size and shape of colonies that contained more cells and were formed by more numerous subcolonies. Also, T cells were found capable of promoting colony formation by HLA-DR\(^+\) non-T cells (Fig 2). However, in experiments in which T cells and HLA-DR\(^+\) non-T cells from the same donor were run simultaneously against autologous HLA-DR\(^+\) target cells, the titration curve of the BFU-E promoting effect of the two cell types appeared somewhat different. Unfortunately, in the present experimental system, a plateau of colonies could not be reached when T cells were added, since co-culture of T cells with HLA-DR\(^+\) non-T cells at a 4:1 ratio resulted in overcrowding of plates. Incidentally, the finding that HLA-DR\(^+\) cells originated burst colonies in the presence of purified T cells represents additional evidence that they contained BFU-E precursors.

**Table 2. Cooperation Between HLA-DR\(^+\) and HLA-DR\(^-\) Non-T Cells for Optimal In Vitro Growth of Erythroid Bursts**

<table>
<thead>
<tr>
<th>Cell Suspension*</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
<th>Experiment 4</th>
<th>Experiment 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfractionated non-T cells</td>
<td>25 ± 3</td>
<td>38.0 ± 4.0</td>
<td>19 ± 3</td>
<td>39.0 ± 5.0</td>
<td>43 ± 6</td>
</tr>
<tr>
<td>HLA-DR(^+) non-T cells</td>
<td>4 ± 1</td>
<td>0</td>
<td>0</td>
<td>1.0 ± 0.2</td>
<td>0</td>
</tr>
<tr>
<td>HLA-DR non-T cells</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HLA-DR(^+) + HLA-DR(^-) non-T cells†</td>
<td>42 ± 6 (21 ± 3)</td>
<td>110 ± 18 (55 ± 9)</td>
<td>68 ± 10 (34 ± 5)</td>
<td>82 ± 12 (41 ± 6)</td>
<td>96 ± 14 (48 ± 7)</td>
</tr>
<tr>
<td>HLA-DR(^+) + HLA-DR(^-) non-T cells</td>
<td>26 ± 3</td>
<td>59.0 ± 7.0</td>
<td>55 ± 4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Irradiated HLA-DR(^-) non-T cells</td>
<td>0</td>
<td>3.0 ± 0.5</td>
<td>5 ± 1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Irradiated HLA-DR(^+) non-T cells</td>
<td>13 ± 2</td>
<td>27.0 ± 3.0</td>
<td>30 ± 4</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*2 × 10^5 cells were plated, with the exception of the experiments where 2 × 10^6 HLA-DR\(^+\) non-T cells were admixed with equal proportion of HLA-DR\(^-\) non-T cells and plated. The 1:1 ratio was selected, considering that non-T cells contained approximately 40% HLA-DR\(^+\) cells (detected values, 35 ± 5% positive cells in three consecutive experiments).

†Results are expressed as mean values ± SEM of triplicate plates.

‡The numbers given in parentheses are the values of colonies calculated per 2 × 10^5 cells plated. This value would allow a better comparison with that obtained with the same number of unfractionated non-T cells plated.

**Characterization of the HLA-DR\(^-\) Non-T Cell Phenotype**

Table 1 summarizes the surface markers, cytochemical features, and NK activities of both HLA-DR and HLA-DR\(^+\) cells and also of Percoll-purified LGLs. The large majority of HLA-DR cells were FcR\(^+\), OKM\(^1\), Leu 7\(^+\) and Leu 11\(^+\). Cells expressing T cell markers were virtually absent, except for a minority of OKT8\(^+\) cells. The surface phenotype of HLA-DR cells, therefore, is similar to that reported for LGL numbers.\(^{33,34,37,38}\) Consistent with the notion that HLA-DR non-T cells were composed mainly of LGLs were the similarities between the surface markers of these cells and those of Percoll-purified LGLs (Table 1). The surface marker analysis of HLA-DR\(^+\) non-T cells demonstrated that they contained mainly monocytes and B cells, a finding that is consistent with previous data\(^{39}\) (Table 1).

When preparations were also stained for the cytochemical localization of acid hydrolases (ANAE or AP) and the cells identified as LGLs, T cells or
monocytes according to the criteria presented in the Materials and Methods section (Table 1). Most of the cells in the HLA-DR non-T cells were LGLs. HLA-DR cells contained mainly monocytes and unstained cells (probably B cells).

As expected from the data in Table 1, HLA-DR non-T cells had a potent NK activity that was comparable to that of Percoll-purified LGLs. Such activity was practically absent from the HLA-DR non-T cells (Table 1).

Identification of the Surface Phenotype of BFU-E Promoting Cells

The observation that LGLs are the preponderant cell type in the HLA-DR non-T cells, suggested that LGLs may have an erythroid burst-promoting activity. This hypothesis was investigated further by assessing directly the surface phenotype of the BFU-E promoting cells. Non-T cells were separated into FcR+ and FcR- cells, and the two fractions were tested for colony formation (Table 3); FcR- cells only formed colonies, although less than that expected from the enrichment in BFU-Es determined by the removal of FcR+ cells (that did not contain BFU-Es). Addition of FcR+ to FcR cells (at a 1:1 ratio) resulted in the formation of a number of colonies comparable to that of unseparated non-T cells (Table 3).

Non-T cells were separated in HLA-DR+ and HLA-DR- cells. HLA-DR+ cells were treated with one of several monoclonal antibodies and complement before being admixed to HLA-DR- cells and tested for BFU-E colony formation. Treatment of HLA-DR non-T cells with Leu 7, Leu 11, OKM1 monoclonals, and complement caused a marked decrease in colony formation. In contrast, such effect was not observed when the same cells were treated with OKT8, OKT4, and complement (Table 4).

The preceding data indicate that the HLA-DR+ non-T accessory cells capable of promoting BFU-E colony formation had an FcR+OKM1, Leu 7, Leu 11+, OKT8, OKT4 surface phenotype, which is consistent with that reported for the majority of LGL numbers.

BFU-E-Promoting Activity of LGLs Purified by Percoll Density Gradient

Low-density cells with morphologic features of LGLs and a strong NK activity can be purified on Percoll density gradients. MNCs were fractionated with this technique, and seven different fractions were

Table 3. Cooperation Between FcR+ and FcR- Non-T Cells in the BFU-E Colony Formation

<table>
<thead>
<tr>
<th>Cell Suspension*</th>
<th>Erythroid Bursts per Plate†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment 1</td>
</tr>
<tr>
<td>Unfractionated non-T cells</td>
<td>17 ± 2</td>
</tr>
<tr>
<td>FcR- non-T cells</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>FcR+ non-T cells</td>
<td>12 ± 1</td>
</tr>
<tr>
<td>FcR+ + FcR- non-T cells</td>
<td>32 ± 4 (16 ± 2) ‡</td>
</tr>
</tbody>
</table>

*2 x 10^6 cells were plated, with the exception of the experiments where 2 x 10^6 FcR- non-T cells were admixed with equal proportion of FcR+ non-T cells and plated. The 1:1 ratio was selected, considering that non-T cells contained approximately 40% FcR+ cells (detected values 40 ± 8% positive cells in three consecutive experiments).
†Results are expressed as mean ± SEM for triplicate cultures.
‡In brackets the values of colonies calculated per 2 x 10^6 cells plated. This value would allow a better comparison with that obtained with the same number of unfractionated non-T cells plated.

Table 4. Cell Surface Phenotype of HLA-DR+ Non-T Accessory Cells as Determined by Cytotoxicity

<table>
<thead>
<tr>
<th>Monoclonal Antibodies and Complement Used to Treat Accessory Cells*</th>
<th>Erythroid Bursts per Plate†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment 1</td>
</tr>
<tr>
<td>C' (control)</td>
<td>36.0 ± 5.0</td>
</tr>
<tr>
<td>Leu 7 + C'</td>
<td>13.0 ± 2.0</td>
</tr>
<tr>
<td>Leu 11b + C'</td>
<td>8.0 ± 1.5</td>
</tr>
<tr>
<td>C' (control)</td>
<td>28.0 ± 4.0</td>
</tr>
<tr>
<td>OKT4 + C'</td>
<td>24.0 ± 2.0</td>
</tr>
<tr>
<td>OKT8 + C'</td>
<td>25.0 ± 3.5</td>
</tr>
<tr>
<td>OKM1 + C'</td>
<td>10.0 ± 1.0</td>
</tr>
</tbody>
</table>

ND, not done.
*2 x 10^6 HLA-DR+ non-T cells were plated in the presence of 2 x 10^6 HLA-DR- non-T cells that had been pretreated with monoclonal antibodies and complement, as described in Materials and Methods.
†Results are expressed as mean ± SEM for triplicate cultures.
SUPERNATANTS appertant from Table 5, collected and tested for their capacity to promote and cultured for 48 hours. The cell supernatants were complement could suggest that this particular LGL capable of promoting BFU-E colony formation in T cells under the same conditions were less efficient in promoting BFU-E colony formation as the HLA-DR non-T cells on Pencoll-purified LGLs. As apparent from Table 5, these supernatants were as efficient in promoting BFU-E colony formation as the LGLs themselves. Supernatants obtained by culturing T cells under the same conditions were less efficient then those obtained by culturing LGLs. However, supernatants with high BFU-E-promoting activity were obtained by culturing T cells with PHA for 48 hours. In the present experiments, monocyte supernatants consistently failed to promote BFU-Es.

DISCUSSION

The present study demonstrates that peripheral blood non-T, non-B cells contain an accessory cell capable of promoting BFU-E colony formation in vitro. This cell has an HLA-DR, FeR, OKM1, Leu 7, Leu 11, OKT4, OKT8 surface phenotype, which is consistent with that of the majority of LGLs. Moreover, Percoll density gradient-purified LGLs proved capable of promoting BFU-E colony formation. These results lead to the conclusion that LGLs are endowed with an erythroid burst-promoting effect. It has been reported recently that a minority of LGLs express the OKT8 instead of the OKM1 marker. The failure to inhibit the accessory function by pretreatment with OKT8 monoclonal antibody and complement could suggest that this particular LGL subset does not promote BFU-E colonies.

The observed promoting effect was not attributable to T cells, B cells, or monocytes. That the accessory cells were not T cells was demonstrated by the following: (1) The accessory cells were negative for the surface markers of the helper/inducer T cells. Recent experiments have demonstrated that this is the T cell subset active in erythropoiesis. (2) The accessory cells had a low density. (3) They were relatively radio-resistant. The BFU-E-enhancing activity of T cells was shown to be abrogated by low-dose irradiation, a finding that was confirmed in our study (data not shown). Finally, although not conclusive for the aforementioned technical reasons, the titration of T cells and LGLs suggested that, in the present experimental system, LGLs were more efficient on a per cell basis than T cells in promoting colony formation. While this article was in preparation, Mangan et al reported that Leu 7 NK cells promote peripheral blood erythroid burst colonies, although Leu 7 NK cells are less efficient on a per cell basis than purified T cells. At present, owing to the differences in the experimental approach used, the reason for these discrepancies cannot be explained.

HLA-DR non-T cells, which were highly enriched for B cells and monocytes, failed to form colonies. This finding excludes the possibility that, in the present experimental system, these two cell types had a BFU-E-promoting activity. Furthermore, cell suspensions with high BFU-E-promoting activity (ie, HLA-DR non-T cells or Percoll-purified LGLs) were virtually devoid of monocytes and B cells. The data on B cells are in agreement with previous findings, whereas those on monocytes are consistent with some, but not all, of the available data. Other investigators have reported that monocytes may exert a promoting or an inhibitory effect on BFU-Es.

One interesting possibility is that LGLs, in order to promote BFU-E colony growth, need the cooperation of monocytes present within HLA-DR cells. This hypothesis, which would reconcile some of the discrepancies between the different studies, is presently under investigation.

The demonstration that LGLs, as well as T cells and macrophages, have a BFU-E-promoting activity raises

<table>
<thead>
<tr>
<th>Culture Supernatant *</th>
<th>Erythroid Bursts per Plate†</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Experiment 1</td>
</tr>
<tr>
<td>None</td>
<td>2.0 ± 0.3</td>
</tr>
<tr>
<td>LGL</td>
<td>24.0 ± 3.0</td>
</tr>
<tr>
<td>Monocytes</td>
<td>0</td>
</tr>
<tr>
<td>T cells</td>
<td>7.0 ± 1.0</td>
</tr>
<tr>
<td>PHA-stimulated T cells</td>
<td>35.0 ± 4.0</td>
</tr>
</tbody>
</table>

*Supernatants, prepared as described in the Materials and Methods section, were used at the final concentration of 10%.
†2 × 10⁵ HLA-DR non-T cells were plated. Results are expressed as mean ± SEM for triplicate cultures.
the problem of the relationship between these cells in the in vitro and possibly in vivo regulation of erythropoiesis. However, with presently available data, the relative importance of the different cells cannot be established.

The experiments reported here have been carried out with autologous cell subset combinations. However, in other tests, we have found that both LGLs and T cells exert comparable BFU-E-promoting effects on both allogeneic and autologous cells. This finding is different from that reported for some T cell subsets that must share a number of HLA antigens with the erythroid precursors in order to regulate erythropoiesis.15

Experiments that must be considered as preliminary suggest that LGLs promote BFU-E colony formation through release of soluble factors. In order to release these factors, LGLs do not appear to require any apparent stimulus, although the possibility that they were activated by contact with FCS or even plastic hardware cannot be dismissed. Clearly, however, interaction with immune complexes was not required for LGLs to be able to exert their colony-promoting activity. This is at variance with that observed for other LGL functions that are mediated only by immune-complex-activated LGLs.29,45

The target cells for the factors released by LGLs are presently unknown, and the problem is currently being investigated together with the biochemical properties of the factors. Of interest in this connection is the observation that cell lines derived from LGLs are potent BPA producers (Pistoia et al, manuscript in preparation).

A number of studies have demonstrated that in man LGLs represent the principal mediators of NK activity.28 Whether or not the same LGLs with cytotoxic functions also have a regulatory activity on hemopoiesis remains to be established. Recent observations in mice using NK cell clones showed that this may be the case.46 The problem is now amenable to investigation in man, since cloned LGLs with NK activity are available.47,49

Previous in vitro experiments using bone marrow cells have demonstrated that NK cells may exert a negative regulatory control on both granulocytopoiesis and erythropoiesis.50-52 These results—together with the present data, which show that NK cells have a promoting effect on circulating BFU-Es—suggest a complex regulatory function of NK cells on hematopoiesis. This concept seems reinforced by observations that excessive inhibition by NK cells may be responsible for clinical conditions characterized by anemia or granulocytopenia.53-55

In some of the studies, the negative control on hemic cell growth could be attributed to direct cell cytolysis on relatively well-differentiated precursors.50,52 In contrast, promotion of cell proliferation may be mediated by soluble factors. These data could suggest the intriguing hypothesis that the same cell that exerts a negative control on cell proliferation through direct cytotoxicity may promote the growth of immature precursors by releasing soluble factors. A mechanism of this kind would ensure a constant surveillance device constituted by the presence of NK cells wherever hemic cells begin to proliferate.

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Large granular lymphocytes have a promoting activity on human peripheral blood erythroid burst-forming units

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